Blazeispirol A from *Agaricus blazei* Fermentation Product Induces Cell Death in Human Hepatoma Hep 3B Cells through Caspase-Dependent and Caspase-Independent Pathways

Zheng-Yuan Su, Yen-Chen Tung, Lucy Sun Hwang, and Lee-Yan Sheen*

Graduate Institute of Food Science and Technology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, 106 Taipei, Taiwan, Republic of China

ABSTRACT: Currently, liver cancer is a leading cause of cancer-related death in the world. Hepatocellular carcinoma is the most common type of liver cancer. Previously, it was reported that blazeispirol A (BA) is the most active antihepatoma compound in an ethanolic extract of *Agaricus blazei* fermentation product. The aim of this study was to understand the antihepatoma mechanism of BA in human liver cancer Hep 3B cells. The results showed that BA inhibited the growth of Hep 3B cells and increased the percentage of cells in sub-G1 phase in a concentration- and time-dependent manner. In addition, BA treatment resulted in DNA fragmentation, caspase-9 and caspase-3 activations, poly(ADP-ribose)polymerase (PARP) degradation, down-regulation of Bcl-2 and Bcl-xL expressions, up-regulation of Bax expression, and disruption of the mitochondrial membrane potential (MMP) in Hep 3B cells. Furthermore, z-VAD-fmk, a caspase inhibitor, did not enhance the viability of BA-treated Hep 3B cells, and BA induced the release of HtrA2/Omi and apoptosis-inducing factor (AIF) from mitochondria into the cytosol. These findings suggested that BA with novel chemopreventive and chemotherapeutic potentials causes both caspase-dependent and caspase-independent cell death in Hep 3B cells.

KEYWORDS: *Agaricus blazei*, blazeisperol A, fermentation product, antihepatoma activity, Hep 3B cells, caspase

■ INTRODUCTION

Agaricus blazei, an edible and medicinal mushroom, possesses antioxidative,[1] antigenotoxic,[2] antitumor/antiangiogenic,[3] antidiabetic,[4] hepatoprotective,[5] and immunomodulatory[6] properties. Currently, fermentation is used to produce large amounts of bioactive fungal products, which have immunomodulatory,[7] antitumor,[8] antioxidative,[9] and antiviral[10] effects. Previously, we demonstrated that an ethanolic extract of *A. blazei* fermentation product decreased the viability of human hepatoma Hep 3B and Hep G2 cells and further isolated a potential antihepatoma compound, blazeispirol A (BA) (Figure 1), from this extract.[11] BA is a blazeispirane derivative, which is a novel group of naturally occurring steroids in *A. blazei* fermented mycelia,[12] and we also found that the production of BA in *A. blazei* fermentation product can be promoted by culturing with black soybean.[11] However, the bioactivities of BA, including its cytotoxic mechanism, have not yet been investigated.

Hepatocellular carcinoma is a prevalent malignancy and is a significant health care problem in the world.[13] Chemotherapy and chemoprevention are used to treat cancer by killing cancer cells and arresting or reversing the tumorigenic process in premalignant cells, respectively.[14] Many chemotherapeutic agents, including natural compounds, have been investigated for their apoptosis-mediated inhibitory effects on cancer cells.[15,16] Recent studies have shown that there are three major apoptotic pathways, namely, the mitochondrial-mediated, death receptor-mediated, and endoplasmic reticulum-mediated pathways.[15,17,18] In the mitochondrial-mediated pathway, chemotherapeutic agents alter the mitochondrial membrane permeability, which results in the release of mitochondrial cytochrome *c*, formation of apoptotic protease activating factor 1/caspase-9 complexes, activation of

caspase-3, and cleavage of poly(ADP-ribose)polymerase (PARP).[19] On the other hand, apoptosis-inducing factor (AIF), a mitochondrial protein, induces large-scale DNA fragmentation after nuclear translocation in a caspase-independent apoptotic pathway.[20] HtrA2/Omi, a mitochondrial serine protease, causes both caspase-independent cell death via its serine protease activity and caspase-dependent apoptosis by binding and inhibiting the cellular inhibitor of apoptosis protein (IAP) family.[21,22]

The aim of this study was to investigate the antihepatoma mechanism of BA in Hep 3B cells. We demonstrated that BA induced apoptosis in Hep 3B cells via loss of the mitochondrial membrane potential (MMP) and increased caspase activation. In addition, treatment with z-VAD-fmk, a caspase inhibitor, conferred mild protection against BA-induced cell death. Immunoblotting with specific antibodies also showed that BA induced cell death through both caspase-dependent and caspase-independent pathways in Hep 3B cells.

■ MATERIALS AND METHODS

Chemicals. Ethanol (95%) was purchased from Echo Chemical (Taipei, Taiwan). Ethyl acetate (Merck, Darmstadt, Germany), *n*-hexane (95%) (Seedchem, Melbourne, Australia), acetic acid, and methanol (Tedia, OH) were used for the isolation of BA. Antibiotic−antimycotic solution, Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acid solution, and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY). 3,3′-Dihexyloxacarbocyanine
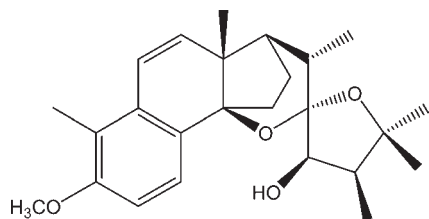
Figure 1. Chemical structure of blazeispirol A (BA).

iodide (DiOC$_6$) and z-VAD-fmk were obtained from Merck. *N,N*-Dimethylfluoramide (DMF) was obtained from Lab-Scan (Dublin, Ireland). Ethylenediaminetetraacetic acid (EDTA) was obtained from Wako Pure Chemical (Osaka, Japan). Aprotinin, benzamidine, digitonin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dithiothreitol (DTT), ethylene glycol bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), leupeptin, 4-nonylphenyl-polyethylene glycol (NP-40), pepstatin, phenylmethanesulfonyl fluoride (PMSF), propidium iodide (PI), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sodium fluoride (NaF), sodium orthovanadate (Na$_3$VO$_4$), sucrose, tris(hydroxymethyl)aminomethane (Tris)-HCl, trypsin−EDTA solution, Triton X-100, and ribonuclease A (RNase A) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Tris/acetic acid/EDTA (TAE) buffer was obtained from Bio-Rad Laboratories (Hercules, CA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Isolation of BA from *A. blazei* Fermentation Product. The *A. blazei* fermentation product was obtained from Prof. Chin-Hang Shu (Department of Chemical and Materials Engineering, National Central University, Taiwan). BA was purified and identified from an ethanolic extract of this fermentation product, as described previously.[11] Briefly, the *A. blazei* fermentation product was extracted with 95% ethanol for 24 h, and then the extract was subjected to silica gel column chromatography and eluted with a stepwise gradient of *n*-hexane/ethyl acetate and ethyl acetate/methanol. The fraction with the highest antihepatoma activity was further purified via semipreparative reverse-phase high-performance liquid chromatography (RP-HPLC). A model L-7100 solvent-delivery system (Hitachi, Tokyo, Japan), a model L-7420 UV−vis detector (Hitachi), and a Peak-ABC chromatography data handling system (JiTeng, Singapore) were used for the HPLC analysis. The fraction was separated on a semipreparative RP-HPLC column (Cosmosil 5C$_{18}$-AR-II, 5 μm, 250 mm × 10 mm i.d.; Nacalai Tesque, Kyoto, Japan) at a column temperature of 25 °C. The mobile phase consisted of methanol/water/acetic acid (89:11:0.5, v/v/v) and was isocratically eluted at a flow rate of 2 mL/min. The effluent was monitored at 243 nm, and BA was collected according to the elution profile. The structure of BA was determined by infrared spectroscopy (Perkin-Elmer 983G spectrophotometer), mass spectrometry (Finnigan MAT-95S mass spectrometer), and ^{1}H and ^{13}C nuclear magnetic resonance (Varia Unity Plus 400 spectrometer, operating in CDCl$_3$ at 400 and 100 MHz, respectively); the structural data were consistent with the literature.[12]

Hepatoma Cell Culture. Hep 3B human hepatoma cells were obtained from the Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan. Cells were cultured in DMEM (pH 7.0) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 100 μM nonessential amino acids at 37 °C, 5% CO$_2$, and 90% relative humidity. Adherent cells were removed from the culture dish with 1 mL of trypsin−EDTA for 3 min at 37 °C.

Cell Cycle Analysis. Hep 3B cells were cultured in 6 cm dishes at a density of 3.25 × 10^5 cells/dish. After 24 h of incubation, the medium was replaced with medium containing various concentrations of BA. Cells were trypsinized with trypsin−EDTA solution at indicated periods of treatment and then collected by centrifugation at 300*g* for 5 min. The

pellet was washed with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol solution, and then stored at −20 °C overnight. After washing with ice-cold PBS and centrifuging (300*g* for 15 min) twice, the fixed cells were resuspended in PBS containing 5% Triton X-100, 2 mg/mL RNase A, and 1 mg/mL PI. The cell cycle of 10000 cells was analyzed by FACScan flow cytometry (BD Biosciences, San Jose, CA). The percentage of cells in the sub-G1 phase was calculated with ModFitLT V3.0 software (Verity Software, Topsham, ME).

DNA Fragmentation Assay. Hep 3B cells (3.25 × 10^5 cells/6 cm dish) were treated with BA at different concentrations. After 48 h of treatment, the cells were harvested and then washed with ice-cold PBS and centrifuged at 300*g* for 5 min. Their DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and electrophoresed in a 2% agarose gel with 0.5× TAE buffer containing 20 mM Tris−acetate and 0.5 mM EDTA (pH 8.0) at 50 V for 50 min. The agarose gel was stained with ethidium bromide (1 μg/mL) and photographed under ultraviolet light (260 nm).

Caspase-3 Activity Assay. Caspase-3 activity in Hep 3B cells was measured as the protease activity of caspase-3, by using PhiPhiLux-G$_2$D$_2$ substrate (OncoImmunin Inc., Gaithersburg, MD) for the cytometric analysis.[23] PhiPhiLux-G$_2$D$_2$ penetrates the cell membrane and is cleaved by caspase-3, which converts it to a fluorescent form. The measurement was performed according to the manufacturer's protocol (Becton Dickinson, San Jose, CA). Briefly, Hep 3B cells (3.25 × 10^5 cells/6 cm dish) treated with BA were harvested, washed with PBS, and centrifuged (300*g* for 5 min) twice. The cell pellet was incubated with 25 μL of PhiPhiLux-G$_2$D$_2$ for 45 min at 37 °C. After a washing with 0.5 mL of flow cytometry dilution buffer, the cells were resuspended in 1 mL of flow cytometry dilution buffer, and then the caspase-3 activity was analyzed by flow cytometry and WinMDI 2.8 software.

MMP Determination. Changes in MMP were examined by flow cytometry by using DiOC$_6$.[24] Hep 3B cells at a density of 3.25 × 10^5 cells/6 cm dish were treated with BA for 48 h. The harvested cells were washed with PBS twice and resuspended with 20 μM DiOC$_6$ at 37 °C for 30 min. The stained cells were detected by flow cytometry, and the mean fluorescence intensity was calculated and compared with that of the untreated cells by using WinMDI 2.8 software.

Preparation of Cell Lysate and Subcellular Fraction. Hep 3B cells (3.25 × 10^5 cells/6 cm dish) were treated with BA at various concentrations. The treated cells were washed with ice-cold PBS and then collected in ice-cold RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.5% NP-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na$_3$VO$_4$, 0.2 mM PMSF, 10 μg/mL leupeptin, and 5 μg/mL aprotinin. The cell lysate was centrifuged at 12000*g* for 20 min to obtain a clear supernatant for analysis.

For subcellular fractionation,[25] the treated cells were washed with ice-cold PBS and then lysed in ice-cold lysis buffer containing 25 mM Tris-HCl (pH 6.8), 0.05% digitonin, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, 10 μg/mL aprotinin, and 10 μg/mL pepstatin with a 26 guage × 0.5 in. needle 10 times. The lysate was centrifuged (750*g* for 5 min) to remove the pellet that contained nuclei and unlysed cells. The residue was centrifuged (10000*g* for 10 min) to obtain a mitochondrial fraction pellet. This pellet was resuspended in gold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, and 10 μg/mL leupeptin. The cytosolic fraction was further isolated from the supernatant by centrifugation (14000*g* for 1 h), which removed the mitochondrial fraction.

Western Blot Analysis. The protein concentrations of the cell lysate and subcellular fraction were measured with a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Samples at the same concentration of protein were subjected to SDS−polyacrylamide gel electrophoresis (SDS-PAGE) with 10−15% gels and then transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, these membranes were hybridized with specific primary antibodies,
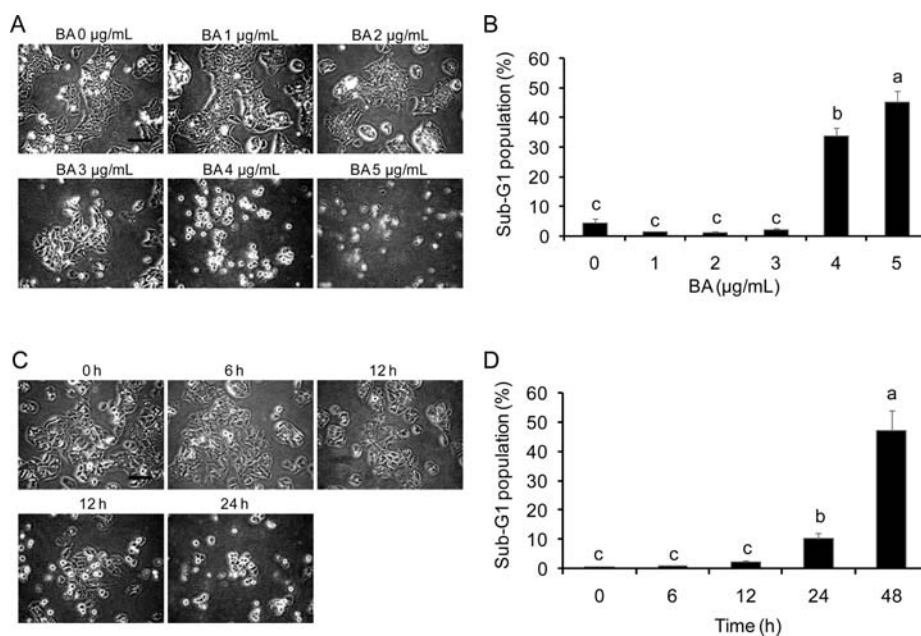
Figure 2. Effect of BA on the growth of human hepatoma Hep 3B cells: (A, B) BA inhibits the growth of Hep 3B cells (A) and increases the percentage of cells in sub-G1 phase (B) in a dose-dependent manner; (C, D) BA inhibits the growth of Hep 3B cells (C) and increases the percentage of cells in sub-G1 phase (D) in a time-dependent manner. Cells were seeded in Dulbecco's modified Eagle's medium (DMEM) in a 6 cm dish for 24 h. Subsequently, cells were treated with various concentrations of BA for 48 h (A, B) or with 4 μg/mL BA for 0–48 h (C, D). Magnification of 200× was used under an inverted-stage microscope equipped with phase contrast (scale bar = 100 μm). The cell population in sub-G1 phase was determined by flow cytometry. Data are expressed as the mean ± SD (n = 3). Numbers not sharing the same letter are significantly different from one another ($p < 0.05$).

including anti-caspase-9, anti-caspase-3, anti-PARP, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-Htr2/Omi, anti-AIF, and anti-β-actin. Either anti-mouse or anti-rabbit IgG secondary antibodies linked to horseradish peroxidase were used to detect the primary antibodies. The antibody-bound proteins were observed by using an enhanced chemiluminescence (ECL) detection kit (General Electric Co., Fairfield, CT).

MTT Assay. Following the methods of You et al.,[26] Hep 3B cells were cultured in 96-well plates at a density of 5 × 10³ cells/100 μL of DMEM per well. After 24 h of incubation, the medium in the 96-well plate was replaced with 50 μL of medium containing 50 μM z-VAD-fmk. After 1 h of treatment, an additional 50 μL of medium containing 50 μM z-VAD-fmk and various concentrations of BA was added. BA and z-VAD-fmk were dissolved in DMSO, and the final concentration of DMSO in the medium was 0.5%. After an additional 48 h of incubation, the medium was discarded, and then 25 μL of MTT solution (5 mg/mL PBS) and 100 μL of medium were added to each well; the cells were reincubated for an additional 4 h. One hundred microliters of MTT lysis buffer (20 g of SDS in 50 mL of DMF and 50 mL of water) were added to dissolve the formazan crystals that formed for 14–16 h. Then, the plates were read at 570 nm on a microplate reader (Anthos 2001, Salzburg, Austria). The cells that were not exposed to treatment served as a control, which showed 100% survival. All analyses were performed in triplicate, and the mean values were determined. The concentration of each sample that was required to inhibit 50% of cell growth (IC$_{50}$) was interpolated from the dose–response curves.

Statistical Analysis. Statistical analyses were performed by using one-way analysis of variance (ANOVA) and Duncan's multiple comparison test (SAS Institute Inc., Cary, NC) to determine statistically significant differences among means ($p < 0.05$).

■ RESULTS

BA Inhibits Cell Viability and Induces Apoptosis in Hep 3B Cells. The morphology of Hep 3B cells treated with different concentrations of BA was examined by inverted-stage microscopy. BA at 0–3 μg/mL inhibited the proliferation of Hep 3B cells as indicated by decreased cell number. BA at 4 or 5 μg/mL induced deformation, atrophy, floating, rupture, and death (Figure 2A). The percentage of Hep 3B cells in sub-G1 phase after treatment with BA for 48 h was determined by flow cytometry. Treatment with 4 or 5 μg/mL of BA significantly increased the percentage of Hep 3B cells in sub-G1 phase by approximately 13- and 18-fold, respectively, compared with control cells that were not treated with BA ($p < 0.05$) (Figure 2B). Hep 3B cells treated with 4 μg/mL BA at continuous time periods displayed morphological changes, including deformation, atrophy, loss of attachment, and rupture (Figure 2C). The percentages of cells in sub-G1 phase were significantly increased from 0.6 to 10.1 and 46.9% after 24 and 48 h of treatment, respectively ($p < 0.05$) (Figure 2D). These data suggested that BA inhibits cell proliferation and induces cell death accompanied with an increase of Hep 3B cells in sub-G1 phase in a dose- and time-dependent manner. Furthermore, Hep 3B cells were treated with 4 μg/mL BA for 24 and 48 h, and the DNA ladder was determined using agarose gel electrophoresis (Figure 3). The results showed that BA caused DNA fragmentation in Hep3B cells in a time-dependent manner compared with the control.

BA Induces Increases in Caspase-9 and Caspase-3 Activities and Cleavage of PARP. Hep 3B cells were treated with different concentrations of BA for 48 h, and the percentage of the cells with active caspase-3 was determined by flow cytometry (Figure 4A). BA at 4 and 5 μg/mL significantly promoted the activation of caspase-3 from 3.31 to 6.81 and 8.53%, respectively ($p < 0.05$). This result suggested that BA-induced apoptosis was related to activation of caspase-3 in Hep 3B cells. After treatment of Hep 3B cells with 4 μg/mL BA for 0, 3, 6, 9, 12, and 24 h, the expression of proteins such as caspase-9, caspase-3, and PARP
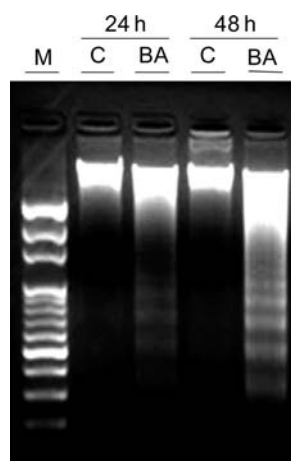
Figure 3. Induction of DNA fragmentation by BA in Hep 3B cells. Cells were treated with 4 µg/mL BA for 24 or 48 h, and DNA fragmentation was analyzed by electrophoresis in a 2% agarose gel. M, DNA marker; C, control; BA, 4 µg/mL BA. A representative result is shown from three separate experiments.

was analyzed by an immunoblot assay (Figure 4B). Caspase-9 and caspase-3 were converted to cleaved products, showing activity after 3 and 6 h, respectively. PARP was also cleaved after 6 h. These results suggested that BA induces caspase-3-dependent apoptosis in Hep 3B cells.

BA Reduces Bcl-2 and Bcl-xL Protein Expression and Increases Bax Protein Expression, Resulting in Mitochondrial Dysfunction. The loss of MMP results in the production of reactive oxygen species and apoptosis.[27] Hep 3B cells were treated with different concentrations of BA for 48 h and then exposed to the fluorescent probe DiOC6, which is taken up by mitochondria, to analyze the change in MMP with a shift in the emission spectrum, using flow cytometry (Figure 5A). A shift of the mean fluorescent intensity to the left from 200.8 to 110.2 showed that MMP was clearly reduced by BA (0−5 µg/mL) in a dose-dependent manner. This result suggested that BA may induce apoptosis in Hep 3B cells through the mitochondria-mediated pathway.

In mitochondria-mediated apoptosis, mitochondrial membrane permeability and MMP were regulated by pro-apoptotic and anti-apoptotic Bcl-2 family proteins.[28] Hep 3B cells were treated with 4 µg/mL BA, and the cytosol was collected at the indicated time points to determine the expression of Bcl-2, Bcl-xL, and Bax proteins by an immunoblot assay (Figure 5B). The results showed that the levels of Bcl-2 and Bcl-xL were decreased after treatment, and their expression was not detected at 24 h of treatment. In contrast, BA increased the protein expression of Bax in a time-dependent manner. This suggested that BA affected the expression of Bcl-2 family proteins, which resulted in high mitochondrial membrane permeability, which causes the release of apoptosis-related factors from mitochondria to cytosol.

BA Causes Translocation of HtrA2/Omi and AIF Proteins from Mitochondria to Cytosol and Leads to Caspase-Independent Cell Death. The aforementioned results suggested that BA induces caspase-dependent apoptosis in Hep 3B cells. We used z-VAD-fmk, a caspase inhibitor, to demonstrate whether BA induced caspase-independent apoptosis. Hep 3B cells were treated with 50 µM z-VAD-fmk for 1 h and then co-incubated with different concentrations of BA (0−5 µg/mL) for an additional 48 h. Except for 2 µg/mL BA treatment, Figure 6A shows that z-VAD-fmk did not significantly increase cell viability
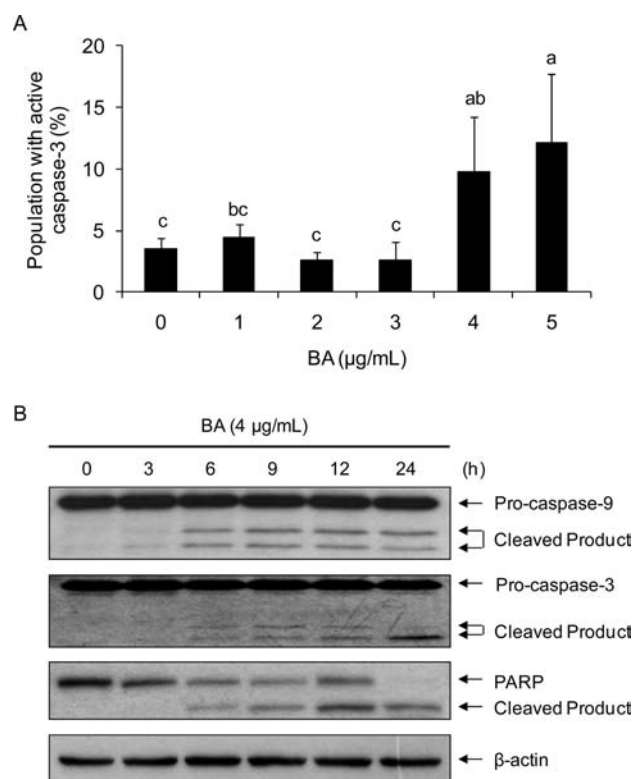

Figure 4. Effect of BA on caspase-9, caspase-3, and poly(ADP-ribose)polymerase (PARP) in Hep 3B cells. (A) BA increases the activity of caspase-3 in Hep 3B cells. Cells were seeded in DMEM in a 6 cm dish for 24 h. Subsequently, the cells were cultured in medium containing various concentrations of BA for an additional 48 h. Then, cells were stained by using the Caspase-3 Kit and analyzed by flow cytometry. Data are expressed as the mean ± SD ($n = 3$). Numbers not sharing the same letter are significantly different from one another ($p < 0.05$). (B) BA induces the cleavage of caspase-9, caspase-3, and PARP in Hep 3B cells. Cells were seeded in DMEM in a 6 cm dish for 24 h. Subsequently, the cells were treated with 4 µg/mL BA for the indicated time periods, and then cell lysates were harvested. Equal amounts of protein from each sample were subjected to Western blotting analysis and probed for caspase-9, caspase-3, and PARP. β-Actin served as a loading control. A representative result is shown from three separate experiments.

compared to the cells treated with the same concentrations of BA, although z-VAD-fmk caused a higher IC$_{50}$ value (2.72 µg/mL) compared with BA-treated cells not treated with z-VAD-fmk (2.51 µg/mL). These results suggested that BA may also induce caspase-independent death in Hep 3B cells.

In caspase-independent cell death, some proteins such as AIF and HrtA2/Omi are released from mitochondria to cytosol.[20,21] After treatment with 4 µg/mL BA for 0−24 h, Hep 3B cells were analyzed by Western blotting for the protein expression of AIF and HrtA2 in cytosolic fractions, which were isolated by using density gradient centrifugation. Figure 6B shows that BA increased the levels of cytosolic AIF and HrtA2/Omi as treatment time increased. This result suggested that BA causes caspase-independent death by inducing translocation of AIF and HrtA2/Omi from mitochondria to cytosol in Hep 3B cells.

■ DISCUSSION

Previously, we showed that BA isolated from an ethanolic extract of *A. blazei* fermentation product effectively inhibits the
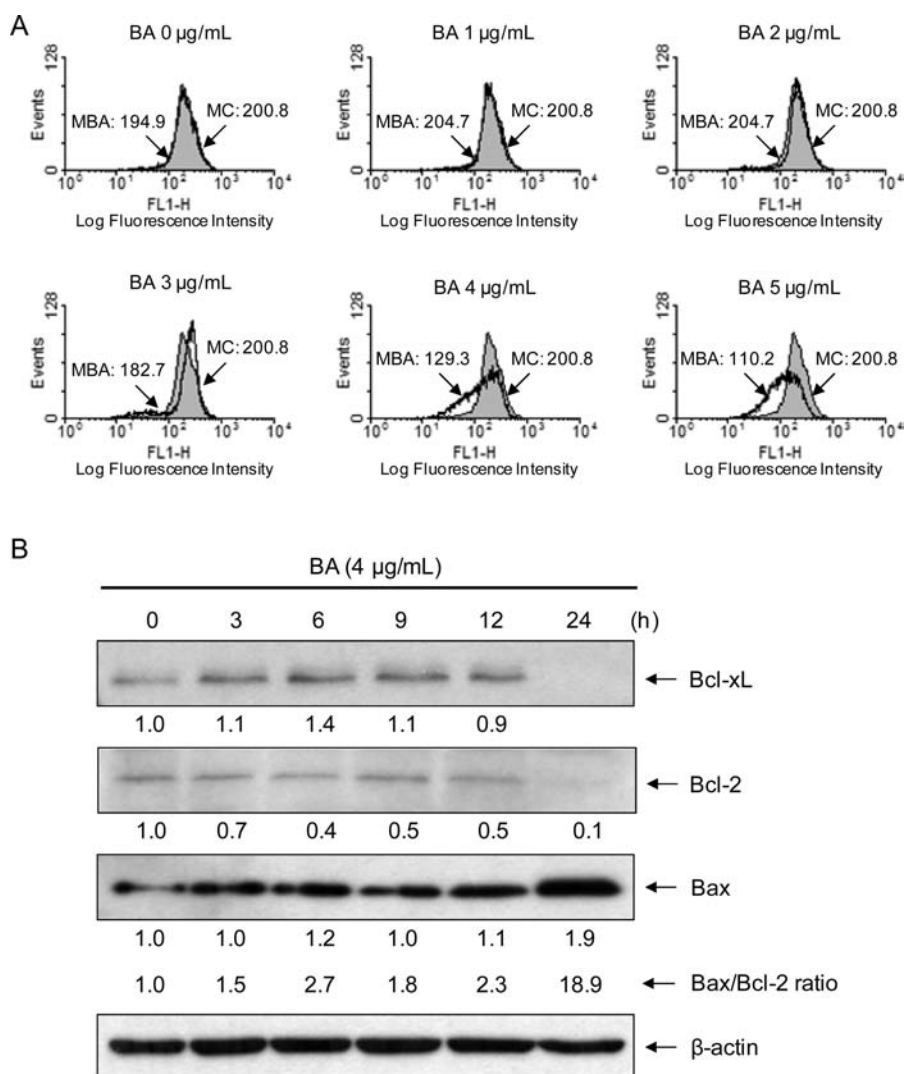
Figure 5. Effect of BA on mitochondrial membrane potential (MMP) and the expression of Bcl-2 family proteins in Hep 3B cells. (A) BA induces loss of MMP in Hep 3B cells. Cells were seeded in DMEM in a 6 cm dish for 24 h. Subsequently, the cells were cultured in medium containing various concentrations of BA for an additional 48 h. Then, the cells were stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and analyzed by flow cytometry. Data are presented as log fluorescence intensity. MC, mean relative fluorescence intensity of the control; MBA, mean relative fluorescence intensity of the BA treatment. (B) BA affects the expression of Bcl-2 family proteins in Hep 3B cells. The cells were treated with 4 μg/mL BA for the indicated time periods, and then the cell lysates were harvested. Equal amounts of protein from each sample were subjected to Western blotting analysis and probed for Bcl-2, Bcl-xL, and Bax. β-Actin served as a loading control. A representative result is shown from three separate experiments.

growth of human hepatoma Hep 3B cells with an IC_{50} of 2.8 μg/mL.[11] This study showed that BA caused morphological alterations in Hep 3B cells in a dose- and time-dependent manner, including shrinkage, flotation, and even dissolution (Figure 2A,C). We further demonstrated that BA induced cell death in Hep 3B cells, as indicated by an increase of the sub-G1 population and DNA fragmentation (Figures 2B,D and 3), which are characteristic of apoptosis.[29] Our results further indicated that BA at 4 μg/mL significantly increased the proportion of Hep 3B cells with caspase-3 activity observed at 48 h of treatment and sequentially induced the cleavages of caspase-9, caspase-3, and PARP after 3 h of treatment (Figure 4). These results suggested that BA causes caspase-dependent apoptosis in Hep 3B cells.

A change in the mitochondrial membrane permeability results in the release of apoptosis factors from mitochondria, which activate caspases.[4,19,30] The Bcl-2 family proteins, including anti-apoptotic proteins such as Bcl-2 and Bcl-xL and pro-apoptotic

proteins such as Bax, have been demonstrated to regulate mitochondrial membrane permeability during the apoptotic process.[28] Excessive expression of Bcl-2 protein blocks drug-induced apoptosis.[31] The protein complex formed with Bcl-xL inhibits apoptosis or maintains the normal condition of mitochondrial outer membrane under stress.[32,33] In addition, Bax aggregates with other apoptotic proteins to make holes in mitochondrial outer membranes and thus increase mitochondrial membrane permeability, resulting in the release of apoptotic factors, such as cytochrome c, from mitochondria to cytosol in the cells that are stimulated by apoptotic signals.[34] The data indicated that BA decreased MMP in a dose-dependent manner, whereas 4 μg/mL BA decreased Bcl-2 and Bcl-xL expression and increased Bax expression in a time-dependent manner (Figure 5). These results suggested that BA regulates the expression of Bcl-2 family proteins, which changes the mitochondrial membrane permeability and results in the loss of MMP.
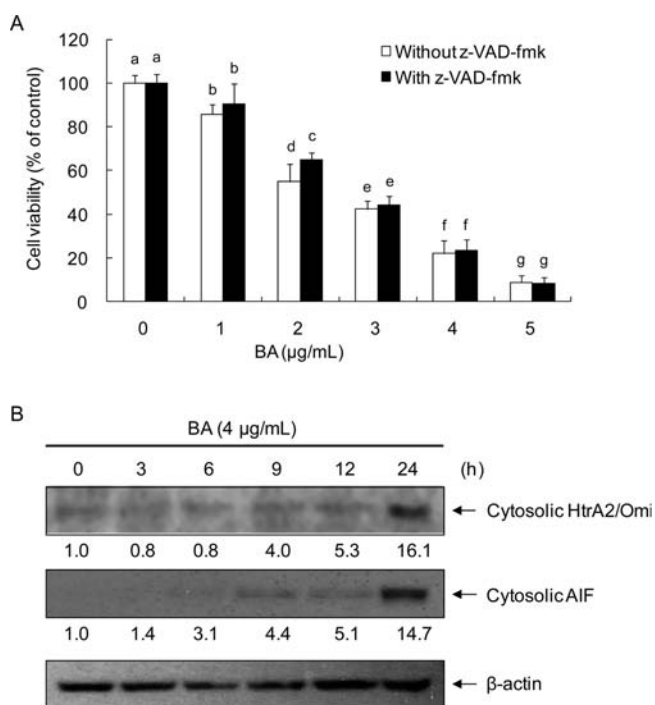
Figure 6. Blazeispirol A induces caspase-independent apoptosis in Hep 3B cells. Panel A shows the effect of BA on the growth of Hep 3B cells pretreated with or without z-VAD-fmk. Cells were seeded in DMEM in a 96-well plate for 24 h. Subsequently, the cells were incubated in medium, pretreated with 50 μM z-VAD-fmk for 1 h, and then incubated with various concentrations of BA for an additional 48 h. Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are expressed as the mean $\pm$ SD (n = 3). Numbers not sharing the same letter are significantly different from one another ($p < 0.05$). Panel B shows the effect of BA on cytosolic HtrA2/Omi and AIF levels in Hep 3B cells. Cells were seeded in DMEM in a 6 cm dish for 24 h. Subsequently, the cells were cultured in medium containing BA (4 μg/mL) for the indicated time periods, and then cell lysates were harvested. Equal amounts of protein from each sample were subjected to Western blotting analysis and probed for HtrA2/Omi and AIF. β-Actin served as a loading control. A representative result is shown from three separate experiments.

Furthermore, BA also decreased the viability of Hep 3B cells pretreated with z-VAD-fmk (Figure 6A). This suggested that BA induced cell death in Hep 3B cells through a caspase-independent pathway. AIF is a flavin protein that plays a critical role in caspase-independent apoptosis.[20] It is released from the mitochondrial intermembranous space to cytosol during apoptotic progress, resulting in DNA segmentation.[35] HtrA2/Omi may be released from mitochondria to decompose members of the IAP family and then separates caspases from XIAP to induce apoptosis; it also induces caspase-independent cell death in mammalian cells due to its serine protease activity.[21,36] The results of this study showed that BA increased the expression of cytosolic AIF and HtrA2/Omi (Figure 6B), which might induce caspase-independent cell death in Hep 3B cells in addition to caspase-dependent apoptosis. However, a more detailed mechanism of BA-induced apoptosis in Hep 3B cells remains to be elucidated. BA-induced apoptosis may be mediated by two pathways: (1) BA may enter into cells and then affect protein expression to induce apoptosis directly, or (2) BA may interact with cell membrane receptors and further affect apoptotic signal transduction. Therefore,
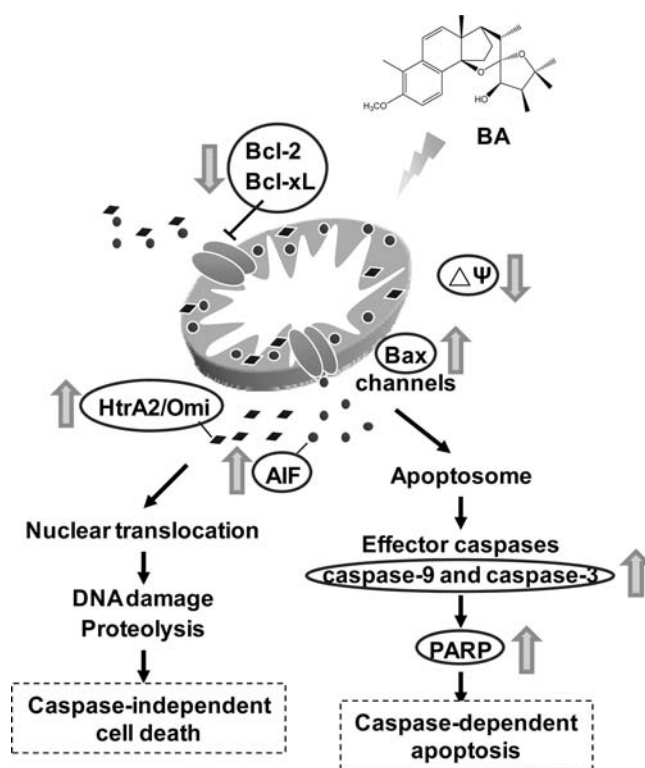

Figure 7. Proposed model of BA-mediated cell death in human hepatoma Hep 3B cells. Arrows indicate the changes in our results.

the mechanism of anticancer activity of BA merits further investigation in future studies.

In conclusion, our results suggested that BA decreases the viability of hepatoma Hep 3B cells by inducing caspase-dependent and caspase-independent cell death as shown in Figure 7. Through caspase-dependent apoptosis, BA decreases Bcl-2 and Bcl-xL expression and increases Bax expression, which leads to a loss of the MMP. Furthermore, downstream regulation of caspase-9 and caspase-3 activation, PARP degradation, and DNA fragmentation are induced sequentially. In addition, BA causes caspase-independent cell death because a BA-induced decrease in cell viability is not blocked by z-VAD-fmk, and HtrA2/Omi and AIF are translocated from mitochondria to the cytosol after treatment with BA. This suggests that BA might be a potential chemopreventive agent for liver cancer.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: 886-2-3366-4129. Fax: 886-2-2362-0849. E-mail: lysheen@ntu.edu.tw.

■ ABBREVIATIONS USED

AIF, apoptosis-inducing factor; BA, blazeisipirol A; IAP, inhibitor of apoptosis protein; IC_{50}, concentration of sample required to inhibit cell growth by 50%; MMP, mitochondrial membrane

potential; PARP, poly(ADP-ribose)polymerase; RP-HPLC, reverse-phase high-performance liquid chromatography.

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